

AWARD NUMBER: W81XWH-13-2-0058

TITLE: Positioning Vascularized Composite
Allotransplantation in the Spectrum of Transplantation

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REPORT DATE: October 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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| REPORT DOCUMENTATION PAGE | | | | Form Approved OMB No. 0704-0188 | |
|---|-----------------------------|------------------------------|--|---|--|
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| 1. REPORT DATE October 2015 | | 2. REPORT TYPE Annual | | 3. DATES COVERED 15 Sep 2014 - 14 Sep 2015 | |
| 4. TITLE AND SUBTITLE Positioning Vascularized Composite Allotransplantation in the Spectrum of Transplantation | | | | 5a. CONTRACT NUMBER W81XWH-13-2-0058 | |
| | | | | 5b. GRANT NUMBER W81XWH-13-2-0058 | |
| | | | | 5c. PROGRAM ELEMENT NUMBER | |
| 6. AUTHOR(S) Wayne W Hancock E-Mail: whancock@mail.med.upenn.edu | | | | 5d. PROJECT NUMBER | |
| | | | | 5e. TASK NUMBER | |
| | | | | 5f. WORK UNIT NUMBER | |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Children's Hospital of Philadelphia, Philadelphia, PA 19104 | | | | 8. PERFORMING ORGANIZATION REPORT NUMBER | |
| 9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012 | | | | 10. SPONSOR/MONITOR'S ACRONYM(S) | |
| | | | | 11. SPONSOR/MONITOR'S REPORT NUMBER(S) | |
| 12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited | | | | | |
| 13. SUPPLEMENTARY NOTES | | | | | |
| 14. ABSTRACT We have continued our studies of the immune mechanisms contributing to rejection of vascularized composite allografts (VCA) in murine models, and how these may be overcome to promote long-term allograft survival. We have now firmly established an orthotopic hind limb VCA model in our lab, and using this orthotopic model, have shown that either of 2 protocols, namely costimulation blockade (CD40L monoclonal antibody plus 2 weeks of rapamycin, RPM), or anti-TCR monoclonal antibody plus 2 weeks of RPM, can each achieve long-term VCA survival without maintenance immunosuppression. We are currently using these approaches to explore mechanistic details. Lastly, we have begun to test whether HDAC targeting may have effects on the VCA survival. | | | | | |
| 15. SUBJECT TERMS allograft rejection, tolerance, costimulation blockade | | | | | |
| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT Unclassified | 18. NUMBER OF PAGES 10 | 19a. NAME OF RESPONSIBLE PERSON USAMRMC |
| a. REPORT Unclassified | b. ABSTRACT Unclassified | c. THIS PAGE Unclassified | | | 19b. TELEPHONE NUMBER (include area code) |

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1. INTRODUCTION

The point of our study is to analyze the immune mechanisms contributing to rejection of vascularized composite allografts (VCA) using murine models, and to try and overcome these immune responses and promote long-term VCA survival.

2. KEYWORDS

Vascularized composite allografts, allograft rejection, tolerance, costimulation blockade

3. OVERALL PROJECT SUMMARY

Our goals for **CY15** were to develop Tasks 3 and 4; Task 3 is to test if peri-transplant immunotherapy will allow long-term VCA survival without development of chronic injury; Task 4 involves testing the ability of Treg-based therapies to promote VCA outcomes. Important progress on Task 3 was achieved, and we have also commenced work on Task 4. This progress was accompanied by moving to the more physiologic (and challenging) orthotopic hindlimb VCA model (examples are shown in **Figure 1**).



Figure 1. Orthotopic hindlimb VCA recipients (BALB/c->C57BL/6 mice).

In the following report, at least 6 transplants/group (BALB/c->C57BL/6) were performed.

3.1 Baseline studies of orthotopic hindlimb VCA survival using conventional immunosuppression

In baseline studies, use of the calcineurin inhibitor (CNI), FK506 (Tacrolimus) increased heterotopic VCA allograft survival from 7-8 days in untreated mice to 50% survival of about 4 weeks ($p < 0.05$) when using Alzet pump delivery of 2 mg/kg/d for 14 days (**Figure 2**). When maintenance immunosuppression was increased to 28 days, at the same dosing, heterotopic VCA survival was markedly increased, with 50% survival to 92 days ($p < 0.01$) (**Figure 2**). By contrast, the same 4 weeks of FK506 led to only a doubling of orthotopic VCA survival, with all grafts rejected by 37 days post-Tx (**Figure 2**).

... Though we have not explored use of higher doses, 4 weeks of therapy was already accompanied by >50% rejection such that longer courses of therapy, at least at the current dosing, is not likely to be useful. Clearly, it is harder to prolong survival of orthotopic vs. heterotopic VCA hindlimb grafts using maintenance immunosuppression. We propose this is because of the markedly increased amount of tissue grafted when using the orthotopic model, though other factors may contribute. Rather than pursuing this point, we moved on to explore the effects of peri-transplant immunomodulation, with the knowledge that the potential barrier to graft acceptance was likely be considerably higher than that faced using the heterotopic model.

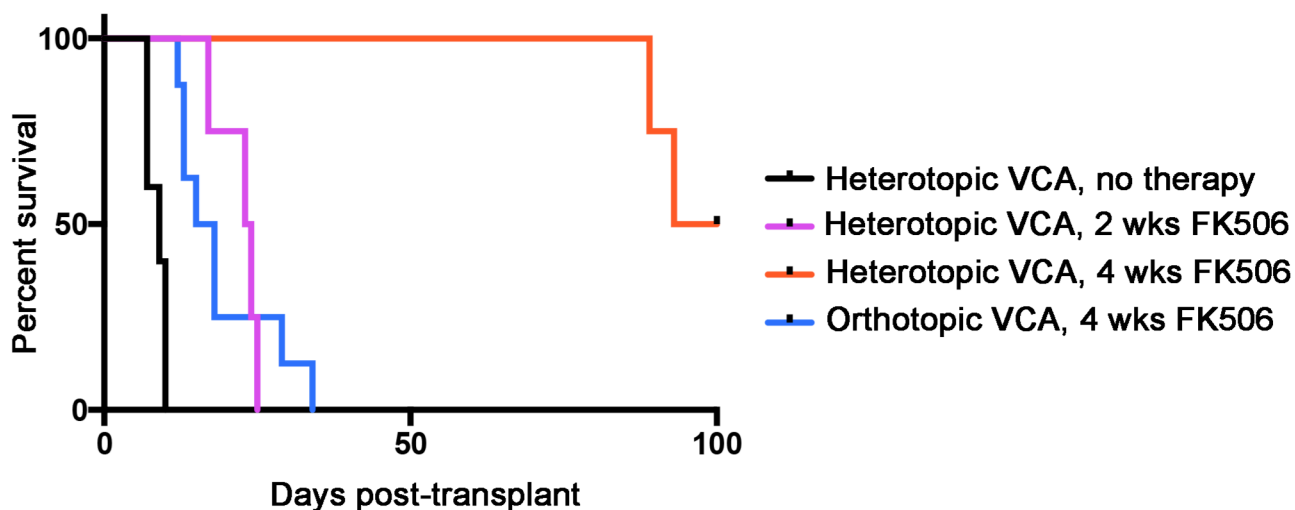


Figure 2. VCA recipients were treated with FK506, delivered by Alzet pumps at a dose of 2 mg/kg/d for the periods specified. In untreated recipients, times to rejection of orthotopic VCA were comparable to that of heterotopic VCA.

3.2 Orthotopic hindlimb VCA survival using CD154/DST plus RPM therapy (Task 3)

Despite the relatively poor efficacy of FK506 in prolonging orthotopic vs. heterotopic VCA grafts, excellent results were seen with CD154 mAb-based costimulation blockade. Thus, a single intravenous injection of CD154 mAb (MR1, 200 µg) and 5×10^6 donor splenocytes (DST), along with 4 weeks of rapamycin (RPM, 2 mg/kg/d, via Alzet pumps), led to 75% long-term orthotopic VCA survival (**Figure 3**) ($p < 0.01$ vs. untreated controls or controls given CD154/DST or RPM alone).

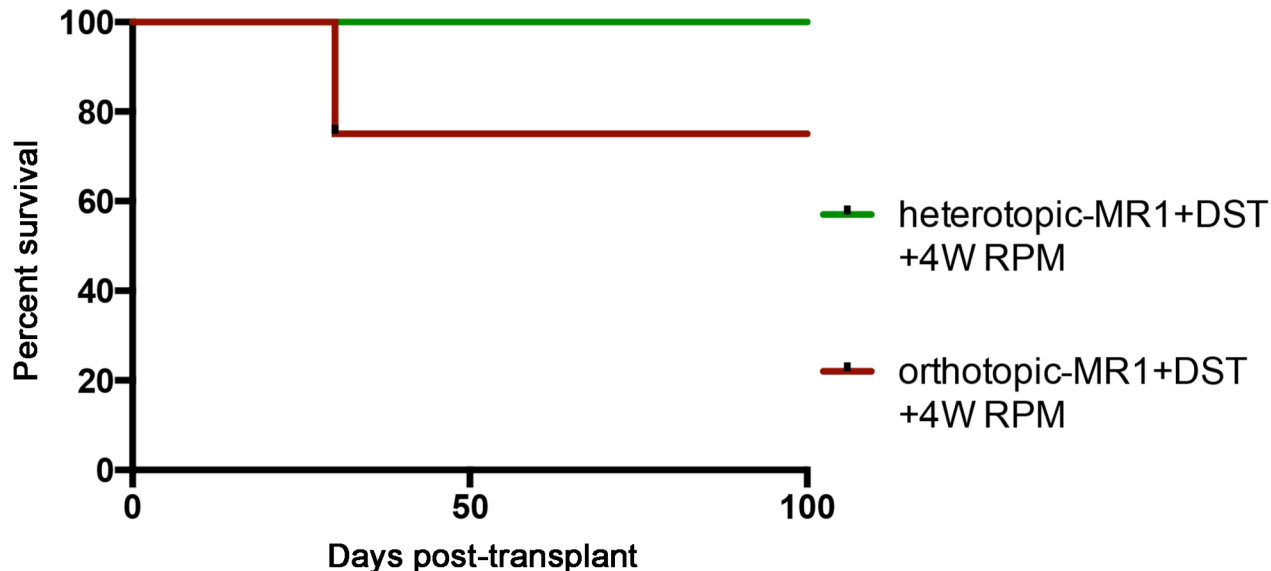


Figure 3. (a) VCA recipients were injected once, i.v., with CD154 mAb (MR1) plus donor splenocytes, at the time of transplantation, and received RPM for 4 weeks thereafter via Alzet pumps; VCA groups are as shown.

... This CD154 mAb-based protocol was almost as effective in orthotopic models as previously noted in our studies using the heterotopic hindlimb VCA model. Historically, while tremendous efficacy was seen using CD154 mAb therapy in rodent transplant models (1), its translation to clinical testing was marred by thromboembolic complications stemming from immune complex crosslinking of FcγRIIIa on human platelets, as reviewed (2, 3). This has led to the development and testing of mAbs directed against CD40, and indeed a group based at Novartis showed a novel, blocking, Fc silent, non-agonistic, and non-depleting anti-CD40

mAb, CFZ533, was able to efficiently prolong the survival and function of kidney allografts in cynomolgus monkeys in the absence of B cell depletion (4). Hence, future studies in non-human primate models might well be directed towards use of such a mAb as CFZ33 or equivalent.

3.3. Orthotopic hindlimb VCA survival using peri-transplant lymphocyte deletion (Task 3)

Induction therapy with polyclonal or monoclonal Abs is widely used in clinical transplantation. Two anti-thymocyte globulin (ATG) preparations are licensed for clinical use in the US for treatment of acute renal allograft rejection, and are used as induction agents before and/or during kidney transplantation. ATG drastically circulating reduces the number of circulating T cells, preventing acute cellular rejection of transplanted organs. Other transplant groups use CD25 (anti-IL-2R) mAb for induction therapy, given its safety profile and specificity for activated T cells. Given this clinical rationale, we have tested the efficacy of a depleting anti-TCR mAb, that we have previously shown was efficacious in murine cardiac allograft recipients (5), in recipients of orthotopic hind-limb allografts.

As noted by our reports during the year, anti-TCR mAb alone (100 µg, qod), when given for 2 weeks from the time of engraftment and then stopped, prolonged heterotopic VCA survival for ~70 days (50% survival data, **red line in Fig. 3**). The addition of 4 weeks of RPM therapy (2 mg/kg/d, Alzet pumps), beginning at the time of transplantation, had no additional useful benefit on heterotopic VCA survival ($p>0.05$) (**green line in Fig. 3**), whereas of the 4 week course of RPM was delayed until 30 days post-Tx, 100% of heterotopic VCA survived for >100 days (**brown line in Fig. 3**). As with FK606 or, to a lesser extent, CD154/DST/RPM therapy, anti-TCR mAb was less effective in prolong VCA survival in orthotopic vs. heterotopic allograft models. However, 50% of orthotopic VCA recipients that also received 4 weeks of RPM from day 30 post-Tx onwards (i.e. “late” RPM) maintained their grafts for >100 days (**purple line in Fig. 3**) ($p<0.01$ vs. untreated recipients or those receiving orthotopic VCA and either TCR mAb alone, or TCR plus RPM from the time of engraftment).

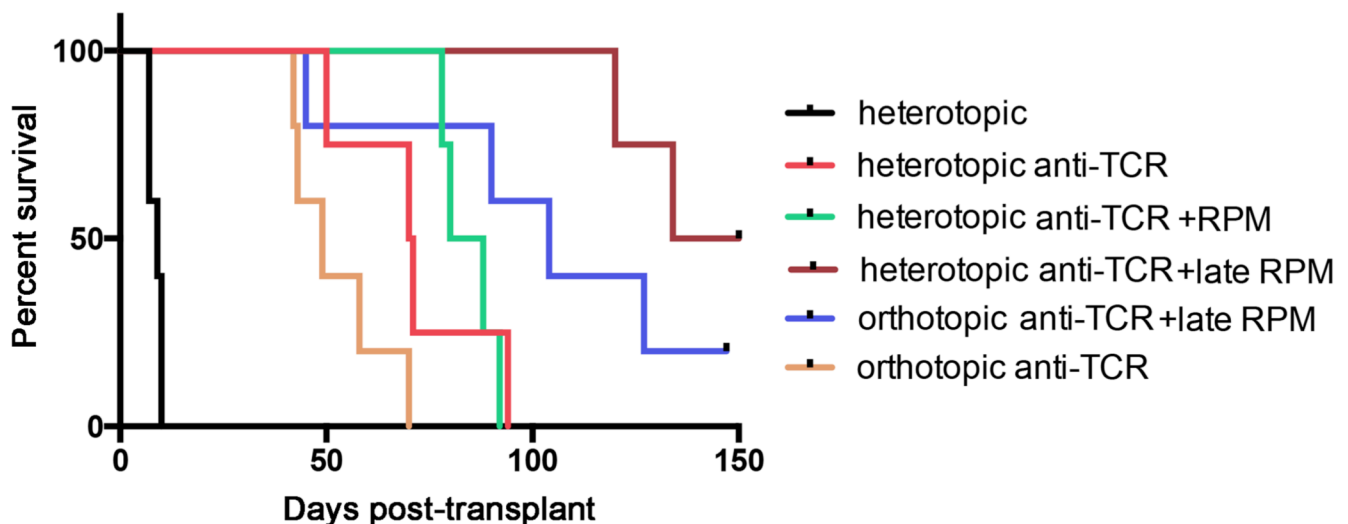


Fig. 3 Effects of anti-TCR mAb (\pm peri-transplant or “late” RPM) on heterotopic vs. orthotopic hindlimb VCA survival.

*** A brief period of T cell depletion and 4 weeks of RPM (from 30 days onwards) produced long-term (>100 d) survival in >50% of orthotopic VCA recipients. Such effects in this stringent strain combination are encouraging, and, as with the CD154 mAb-based project, have translational potential, given the widespread use of “induction” therapy in organ transplant recipients.

3.4 Initial CTLA4Ig plus RPM studies (Task 4)

Using the heterotopic screening model, we have re-examined the effects of CTLA4Ig, previously tested in conjunction with CD154 mAb. Using the protocol of DST at the time of transplantation plus 1 dose of CTLA4Ig at day 2, previously successful in cardiac and renal allograft studies in rodents (6-8), had no beneficial effect of VCA

survival (**yellow line, Fig. 4**), whereas 3 doses of CTLA4Ig plus DST extended 50% survival to about 3 weeks ($p<0.05$) (**blue line, Fig. 4**). Addition of RPM (2 mg/kg/d, 4 weeks, Alzet pumps) markedly improved survival, with 75% of grafts surviving >100 days ($p<0.01$) (**green line, Fig. 3**). With an eye to clinical translation, this led us to test the effects CTLA4Ig plus RPM (but without DST); so far all recipients have maintained their VCA for 30 days ($p<0.01$ vs. CTLA4Ig+DST) (**purple line, Fig. 4**).

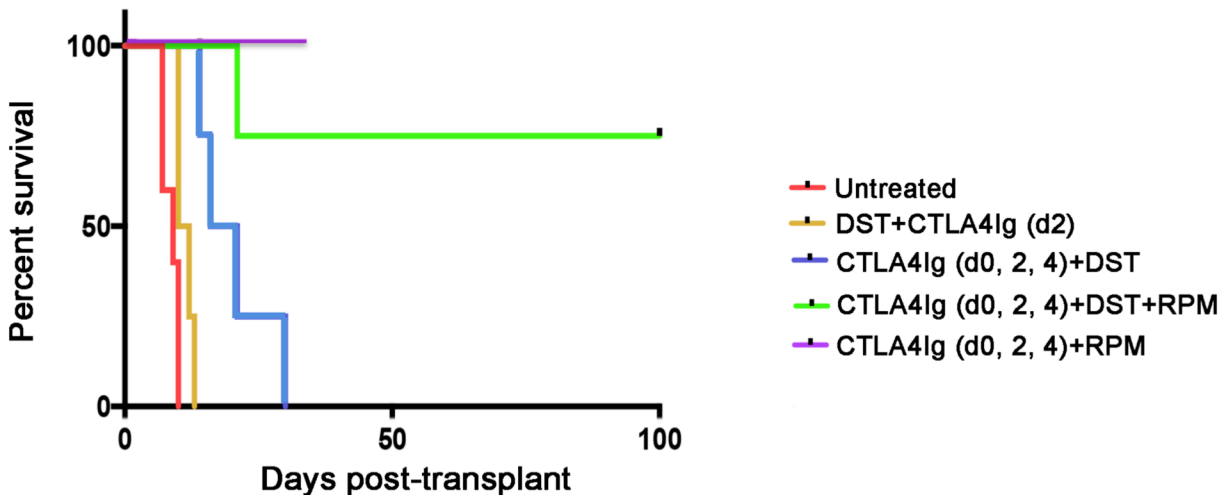


Fig. 4 Effects of CTLA4Ig±DST±RPM on heterotopic VCA survival.

... We now have a third way in which peri-transplant therapy can lead to long-term VCA survival. The survival of recipients treated with CTLA4Ig plus RPM (without DST) will be monitored closely, since CTLA4Ig is clinically approved for renal transplantation (known as Belatacept). Like with CD40 mAb and RPM, and anti-TCR mAb & RPM, CTLA4Ig & RPM may eventually warrant testing in non-human primate models, as is possible in collaboration with other members of our Consortium.

3.5 Initial Treg-oriented studies (Task 4)

With regard to Task 4, involving Treg-oriented studies, we have begun by testing the effects of the pan-HDAC inhibitor, Trichostatin A (TsA) in conjunction with RPM in VCA recipients. This combination was previously shown to be remarkably effective in promoting donor-specific cardiac allograft tolerance, in a Treg-dependent manner, in this strain combination (9). As shown in **Fig. 5**, the combination of TsA (1 mg/kg/d, 14 d) and RPM (2 mg/kg/d, 28 d) prolonged allograft survival ($p<0.05$), but with 50% survival of 3 weeks, the results were not very impressive.

... Task 4 has begun, and we will continue to explore HDAC inhibitor-based protocols in efforts to extend VCA survival.

4. KEY RESEARCH ACCOMPLISHMENTS

- We now have considerable experience with the orthotopic model of hindlimb VCA, which has proven to be a more rigorous test of immunomodulatory therapies than was the corresponding heterotopic VCA model (as seen with the contrasting effects of 4 weeks of FK506 therapy on the survival of orthotopic vs. heterotopic VCA grafts).

- We now have 3 strategies to achieve long-term VCA survival, including 2 (CD154mAb/DST/RPM and anti-TCR/late RPM) that were successful in the orthotopic model, whereas CTLA4Ig/RPM has testing is still underway in the heterotopic model. All 3 approaches have variants that may ultimately be clinically applicable.

- Exploration of Treg-based strategies (Task 4) has begun.

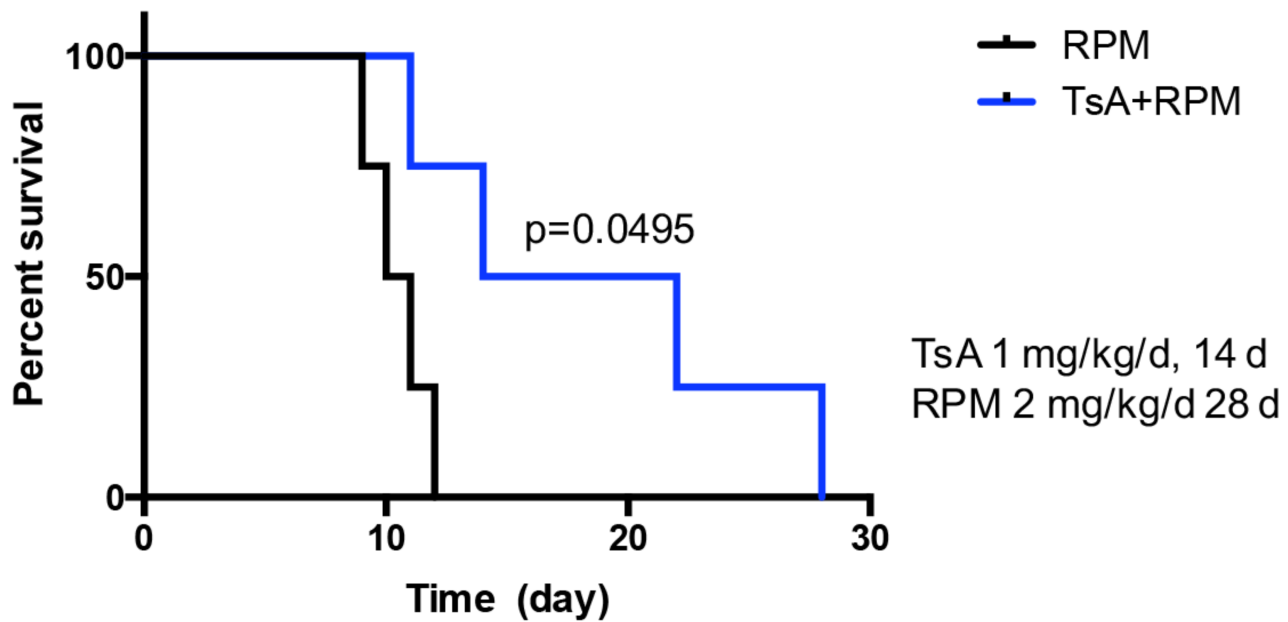


Fig. 5. Initial testing of a pan-HDACi (TsA) plus RPM therapy in heterotopic VCA recipients.

5. CONCLUSIONS

Long-term orthotopic VCA survival is possible with brief peri-transplant therapy and without maintenance immunosuppression. We need to (i) test whether these animals are tolerant of second donor grafts but capable of rejecting third party grafts using this approach; and (ii) assess the role of Tregs in this long-term outcome.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

4 abstracts were presented at 2 scientific meetings and will likely form the nuclei of 2 forthcoming papers:

Wang L, Huang J, Han R, Bhatti TR, Wang Z, Levin LS, Levine MH, **Hancock WW**.

Brief peri-transplant therapy with CD40L, donor splenocyte transfusion and rapamycin causes long-term hindlimb vascularized composite allograft (VCA) survival without a need for maintenance immunosuppression.

12th Congress of the International Hand and Composite Tissue Allotransplantation Society
Philadelphia, PA, April 2015

Wang L, Huang J, Han R, Bhatti TR, Wang Z, Levin LS, Levine MH, **Hancock WW**.

Use of a clinically relevant T cell depletion strategy (anti-TCR mAb) to promote long-term hindlimb vascularized composite allograft (VCA) survival

12th Congress of the International Hand and Composite Tissue Allotransplantation Society
Philadelphia, PA, April 2015

Wang L, Samanta A, Huang J, Bhatti TR, Wang Z, Han R, Levin LS, Levine MH, **Hancock WW**.

Peritransplant costimulation blockade and rapamycin therapy induces permanent limb allograft survival.

American Transplant Congress
Philadelphia, May 2015

Wang L, Samanta A, Huang J, Bhatti TR, Wang Z, Han R, Levin LS, Levine MH, **Hancock WW**.

Clinically relevant anti-TCR mAb induction plus brief rapamycin therapy can achieve long-term vascularized composite allograft (VCA) survival.

American Transplant Congress
Philadelphia, May 2015

7. INVENTIONS, PATENTS AND LICENSES

None.

8. REPORTABLE OUTCOMES

None

9. OTHER ACHIEVEMENTS

None.

10. REFERENCES

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11. APPENDICES

Revised Quad Chart.

Positioning Vascularized Composite Allotransplantation in the Spectrum of Transplantation

CRMRP-JPC8, "Novel Immunomodulatory Therapies for Vascularized Composite Allotransplantation" MR120023P3



PI: Wayne W. Hancock

Org: Children's Hospital of Philadelphia & University of Pennsylvania

Award Amount: \$1,996,875

Study Aims

- Establish murine hindlimb transplant models
- Target chemokine/chemokine receptor pathways promoting VCA rejection
- Test if costimulation blockade will promote long-term VCA survival
- Test if Foxp3+ Treg-directed therapies will promote long-term VCA survival
- Test optimal combinations of therapies so as achieve VCA engraftment and function, as well as preventing development of chronic injury

Approach

Our combined group recognizes that the long-term effects of chronic immunosuppressive therapies, including increased rates of nephrotoxicity, atherosclerotic disease, diabetes and tumor formation, outweigh their usefulness in VCA recipients. To identify less toxic and more suitable therapies for management of VCA, the group will undertake basic science studies in murine models to elucidate the mechanisms of immune rejection of VCA, and test the efficacy of novel strategies to achieve long-term engraftment without use of maintenance immunosuppressive therapy.



Examples of orthotopic hind-limb (stringent BALB/c->C57BL/6 model). • **Ultimately**, we wish to achieve long-term survival and function of orthotopic hind limb allografts in mice (BALB/c->C57BL/6). • **Accomplishment**: in our second year, we have achieved this for orthotopic hindlimb allografts (**photograph**), using peri-operative therapies that are less toxic than the current types of maintenance immunosuppression used in organ transplant recipients.

Timeline and Total Costs (includes direct & indirect costs)

| Activities | 2013 | 2014 | 2015 | 2016 |
|---|-------------|-------------|-------------|-------------|
| Task 1. Obtain regulatory approval and establish murine hindlimb models at CHOP | <div></div> | <div></div> | <div></div> | <div></div> |
| Task 2. Target key chemokine/chemokine receptor pathways promoting VCA rejection. | <div></div> | <div></div> | <div></div> | <div></div> |
| Task 3. Test if peri-transplant costimulatory blockade will allow long-term VCA survival without development of chronic injury. | <div></div> | <div></div> | <div></div> | <div></div> |
| Task 4. Test ability of T-regulatory (Treg) based therapies to promote VCA outcomes. | <div></div> | <div></div> | <div></div> | <div></div> |
| Task 5. Test optimal combinations of therapies based on data generated above. | <div></div> | <div></div> | <div></div> | <div></div> |
| Estimated Budget (total \$K) | 511,875 | 495,000 | 495,000 | 495,000 |

Updated: October 14, 2015

Goals/Milestones

- ✓ **CY13 Goal** – We have established a VCA model and begun chemokine targeting (Task 1);
- ✓ **CY14 Goals** – We continuing chemokine/receptor targeting (Task 2), and using costimulation blockade, we have achieved considerable success (long-term engraftment with brief peri-Tx therapy);
- CY15 Goal** - Complete costimulation blockade & Treg studies (Tasks 3 & 4)
- CY16 Goal** - Undertake final studies using optimal combinations (Task 5)

Comments/Challenges/Issues/Concerns

- If timelines change, comment here: no comments
- If off by more than 1 quarter, comment here: no comments

Budget Expenditure to Date

Projected Expenditure: As budgeted
Actual Expenditure: As budgeted